COMPARATIVE BIOCHEMISTRY OF *p*-HYDROXYMETHYL-L-PHENYLALANINE: IN VIVO STUDIES

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1. Introduction

Previous published results [1–8] proved that both aniline and benzene are enzymatically hydroxymethylated by liver microsomal and bacterial systems and furthermore, the aryl hydroxymethyl compounds form the hydroxylated end products via a hydroxylative–dehydroxymethylation pathway. More recently it was shown that *Escherichia coli* also hydroxymethylates L-phenylalanine to form *p*-hydroxymethyl-L-phenylalanine [9]. It had also been proven that the microorganism forms L-tyrosine via the hydroxylative–dehydroxymethylation reaction and that the organism also forms L-phenylalanine from the aryl hydroxymethyl amino acid [8,9].

It is the purpose of this publication to show that *E. coli* and Gram positive nutritionally fastidious microorganisms metabolize *p*-hydroxymethyl-L-phenylalanine to either phenylalanine or tyrosine. The utilization of the hydroxymethyl amino acid to form tyrosine represents a hydroxylative-dehydroxymethylation reaction. However the rat is incapable of metabolizing the amino acid (*p*-hydroxymethyl-L-phenylalanine).

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2. Materials and methods

The synthesis of the aryl amino acid, *p*-hydroxymethyl-L-phenylalanine and the purification of the tritiated compound were previously described [8]. The specific activity of [^{3}H]*p*-hydroxymethyl-L-phenylalanine was diluted to contain 6 μCi per 1.0 ml that contained 7.0 μM of the amino acid. This solution 0.5 ml (10^6 cpm) was injected intraperitoneally into rats.

Four male albino rats of the Wistar strain (140–150 g) were used for each experiment. Each determination was done in duplicate for each sample of organ, protein, urine, CO_2 and plasma.

Uniformly labeled L-[^{14}C]phenylalanine (Amersham-Searle), 1 mg in 0.5 ml, (8.6×10^5 cpm) was injected i.p. into rats.

Determinations of the expired labeled CO_2, of the excretion of label in the urine and of the incorporation of label into organ and plasma proteins were performed by published techniques [10]. Liquid scintillation counting of all samples were performed by the techniques previously described [10].

The following microorganisms were utilized in these studies: *Escherichia coli* F ATCC* 9723 (the

* American Type Culture Collection.

phenylalanineless mutant); *Leuconostoc mesenteroides* ATCC 8042; *Lactobacillus casei* ATCC 7469; *Lactobacillus leichmannii* ATCC 7830. The medium used to determine the utilization of hydroxymethyl-L-phenylalanine in lieu of phenylalanine by *E. coli* F was that described by Anderson [12].

The medium used to determine the utilization of the aryl hydroxymethyl amino acid by *L. mesenteroides* was that described by Steele et al. [13] deleting either L-phenylalanine or L-tyrosine. Similar techniques were used with *L. casei* [14] and *L. leichmannii* [15]. All incubations were carried out at 37°C for 48–72 hr.

3. Results and discussion

3.1. *Retention of [^{3}H]p-hydroxymethyl-L-phenylalanine in organs of the rat*

Studies on the metabolism of [^{3}H]*p*-hydroxymethyl-L-phenylalanine are summarized in table 1. The retention of label by rat organs, and the loss of the tritiated compound from that organ varies considerably. Two hours after injection the kidney shows greater than five times the count of the liver per gram of tissue, and the pancreas contained greater than three times the number of counts found in the liver. However, 24 hr after the injection the pancreas contained 46% of the counts shown after 2 hr, whereas the loss of label by both the kidney and liver was more substantial from the second to the twenty-fourth hours; after 24 hr, the kidney contained 18% of the label shown at 2 hr, and the liver, 38% of label found at 2 hr. The maintenance of over 50% of the label in the plasma after 24 hr was probably due to leaching of the label from the organs. After 24 hr over 40% of the injected label was excreted into the urine.

Table 1
Retention of radioactivity in organs and plasma and excretion of radioactivity in urine

	2 hr	24 hr
Liver*	2495 ± 33	940 ± 61
Pancreas*	8950 ± 170	4160 ± 62
Kidney*	13,970 ± 280	2562 ± 43
Plasma**	1898 ± 74	995 ± 81
Urine†	18.0 ± 4.5	42.5 ± 1.5

* Counts per minute per g tissue, mean S.D.
** Counts per minute per ml of plasma.
† Cumulative percentage of administrated dose excreted.

3.2. *Studies on the incorporation [^{3}H]hydroxymethyl-L-phenylalanine in rat tissue proteins*

The retention of tritium label in the organs varied considerably (table 1), however the counted radioactivity in these organs did not represent amino acid incorporation into tissues proteins as determined by the technique of Godin and Dolan [10]. Two hours after injection the counts present in tissue proteins (0.2 to 2.0 counts/min/mg protein) were of the order of 0.1–0.5% of those obtained upon injection of L-[^{14}C]phenylalanine. It was shown earlier than when [^{14}C]*p*-fluorophenylalanine was injected into rats, 54% of the radioactivity found in proteins (39–114 counts/min/mg protein) was present as [^{14}C]tyrosine [11]. If the *p*-hydroxymethyl-L-phenylalanine was transformed into phenylalanine or tyrosine by the rat, much more radioactivity would be found in the rat tissue proteins. Furthermore, the in vivo incorporation of [^{14}C]phenylalanine or [^{14}C]tyrosine was not affected by the simultaneous injection of unlabelled *p*-hydroxymethyl-L-phenylalanine. The aryl hydroxymethyl amino acid does not inhibit protein synthesis whereas β-thienyl-alanine does. In addition when β-thienylalanine or 2-amino-3-phenylbutanoic acid were injected simultaneously with [^{14}C]phenylalanine, there was an increase excretion of ^{14}C in the respiratory CO_2 [10]. When *p*-hydroxymethyl-L-phenylalanine (15 mg per 100 g) was injected simultaneously with radioactive phenylalanine or radioactive tyrosine no changes were observed in the rate of excretion and in the quantity of ^{14}C excreted in the CO_2 during the first 6 hr following the injection.

3.3. *Utilization of p-hydroxymethyl-L-phenylalanine by microorganisms*

The utilization of *p*-hydroxymethyl-L-phenylalanine (L-HMPhe) by the phenylalanineless mutant of *E. coli* F was described previously [8] but the data was not quantitated. However publication [8] did prove that the wild strain of *E. coli* (ATCC 9723)

converted L-HMPhe into tyrosine, in the presence of the tyrosine inhibitor, 2 aminotyrosine. This metabolic conversion represented a hydroxylative–dehydroxymethylation reaction. L-HMPhe was shown to be 2% as effective as L-tyrosine under the conditions reported. Therefore, nutritionally fastidious Gram positive organisms were studied to determine whether they were also capable of utilizing the L-isomer of HMPhe in lieu of phenylalanine and tyrosine. The results presented in table 2 conclusively demonstrate that *E. coli, L. mesenteroides, L. casei* and *L. leichmannii* are each capable of metabolizing L-HMPhe to (a) phenylalanine by the cleavage of the C–C bond between the phenyl ring and the hydroxymethyl side chain, and (b) to tyrosine by the hydroxylative–dehydroxymethylation reaction, via a direct hydroxyl replacement of the side chain hydroxymethyl group. The inability of these organisms to utilize phenylalanine per se to satisfy the tyrosine requirement is evidence that the enzyme phenylalanine hydroxylase is absent from these microorganisms. However, Guroff and Ito [16] reported that a species of *Pseudomonas* does possess the phenylalanine hydroxylase.

It is interesting to note that the least nutritionally complex organism (*E. coli*) utilized L-HMPhe ten times more efficiently than the nutritionally complex microorganisms, *L. casei* and *L. leichmannii,* whereas the organism *L. mesenteroides* of intermediate nutritional complexity utilized L-HMPhe four times more effectively than the former two organisms. Furthermore, each species of organism utilized L-HMPhe to form either phenylalanine or tyrosine to essentially the same degree; these data (table 2) demonstrate that the progressive loss of enzymatic function is the same for each enzyme within each species.

Table 2
Microbial utilization of *p*-hydroxymethyl-L-phenylalanine to L-phenylalanine and L-tyrosine

Organism	Percent conversion of *p*-hydroxymethyl-L-phenylalanine to	
	Phenylalanine	Tyrosine
E. coli	1.60	2.00*
L. mesenteroides	0.80	0.80
L. casei	0.18	0.20
L. leichmannii	0.20	0.20

* Data reported by Smith and Sloane [8].

Table 3
Reversal of growth inhibition of *p*-hydroxymethyl-D,L-phenylalanine by the L-isomer

	Absorbance at 600 nm	
	25 hr	42 hr
1. Basal medium	0	0
2. 1 + 2 μmoles D,L-HMPhe	0	0
3. 1 + 4 μmoles D,L-HMPhe	0	0
4. 1 + 4 μmoles L-HMPhe	0.19	–
5. 1 + 4 μmoles L-HMPhe + 4 μmoles D,L-HMPhe	0.18	–
6. 1 + 4 μmoles L-HMPhe + 2 μmoles D,L-HMPhe	0.17	–
7. 1 + 2 μmoles L-HMPhe	–	0.50

E. coli F was used in these studies; the cultures were incubated at 37°C as previously described [8].

The inability of the D,L-isomer to support the growth of these microorganisms may be due to the interference of the uptake of the L-isomer by the D-isomer. The addition of 2 μmoles of the L-isomer to the medium that continued 4 μmoles of the D,L-isomer allowed a growth response by *E. coli* after 25 hr incubation that was maximal for this level of L-HMPhe. After 42 hr incubation the organisms did not show a growth response to the D,L-isomer. Similar results were obtained with *L. mesenteroides, L. casei,* and *L. leichmannii,* utilizing 10 μmoles of the D,L-compound and 10 μmoles of the L-isomer. The data with *E. coli* are presented in table 3.

The data presented in this paper demonstrate that the rat is incapable of metabolizing L-HMPhe to phenylalanine or tyrosine, whereas microorganisms metabolize L-HMPhe to (a) phenylalanine and (b) tyrosine by a pathway that does not involve hydroxylation of phenylalanine per se. Thus these pathways are not unique to *E. coli* (a question posed by Umbarger [17]) but these pathways do not appear to be significant ones since the hydroxymethylation of L-phenylalanine by *E. coli* occurs to only a limited extent [9].

The concomitant hydroxylative rupture of a

C–C bond between the phenyl ring and the side chain carbon atom has also been shown to occur in the formation of catechol from salicylic acid [18] and in the biosynthesis of coenzyme Q [19].

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References

[1] Sloane, N. H. (1961) J. Biol. Chem. 236, 448–454.
[2] Sloane, N. H., Untch, K. G. and Johnson, A. W. (1963) Biochim. Biophys. Acta 78, 558–593.
[3] Sloane, N. H. and Untch, K. G. (1962) Biochim. Biophys. Acta 59, 511–512.
[4] Sloane, N. H. and Untch, K. G. (1964) Biochemistry 3, 1160–1164.
[5] Sloane, N. H. (1964) Biochim. Biophys. Acta 81, 408–410.
[6] Sloane, N. H. (1965) Biochim. Biophys. Acta 107, 599–602.
[7] Sloane, N. H. and Heinemann, M. (1967) Biochim. Biophys. Acta 141, 47–54.
[8] Smith, S. C., and Sloane, N. H. (1967) Biochim. Biophys. Acta 148, 414–422.
[9] Sloane, N. H. and Smith, S. C. (1968) Biochim. Biophys. Acta 158, 394–401.
[10] Godin, C. and Dolan, G. (1967) Canad. J. Biochemistry 45, 71–79.
[11] Dolan, G. and Godin, C. (1966) Biochemistry 5, 922–925.
[12] Anderson, E. H. (1946) Proc. Natl. Acad. Sci. U.S. 32, 120–128.
[13] Steele, B. F., Sauberlich, H. E., Reynolds, M. S. and Baumann, C. A. (1949) J. Biol. Chem. 177, 533–544.
[14] Flynn, L. M., Williams, V. B., O Dell, B. D. and Hogan, A. G. (1951) Anal. Chem. 23, 180–185.
[15] Thompson, H. T., Dietrich, L. S., and Elvehjem, C. A. (1950) J. Biol. Chem. 184, 175–180.
[16] Guroff, G. and Ito, T. (1963) Biochim. Biophys. Acta 77, 159–161.
[17] Umbarger, H. E. (1971) Advances in Genetics 16, 119–140.
[18] Katagiri, M., Yamamoto, S. and Hayaiski, O. (1962) J. Biol. Chem. 237, 2413–2414.
[19] Rudney, H. and Parson, W. W. (1963) J. Biol. Chem. 238, 3137–3138.